Fluorogenic L-alanylaminopeptidase substrates derived from 6-amino-2-hetarylquinolines and 7-amino-3-hetarylcoumarins and their potential applications in diagnostic microbiology

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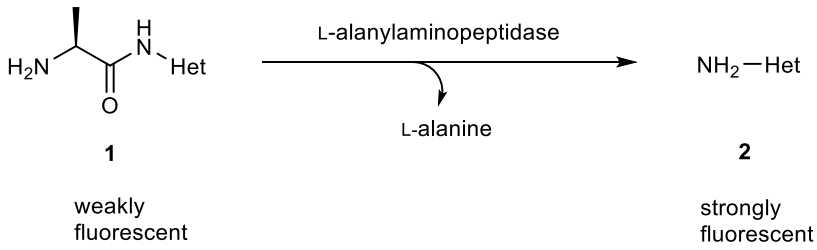
Abstract. Six novel fluorogenic enzyme substrates for detecting L-alanylaminopeptidase activity in microorganisms have been prepared and evaluated in Columbia agar media. The substrates are L-alanyl derivatives of 6-amino-2-hetarylquinolines and 7-amino-3-hetarylcoumarins. Both the quinoline and coumarin series of substrates produced fluorescence in the presence of Gram-negative microorganisms. In contrast, fluorescence generation in the presence of the Gram-positive microorganisms and yeasts was limited or absent.

Keywords: Fluorogenic substrates, L-alanylaminopeptidase, microorganism detection, quinolines, coumarins

The detection of L-alanylaminopeptidase activity in microorganisms often provides a useful insight as to whether a microorganism of interest can be categorised as either Gram-negative or Gram-positive. This is because Gram-negative microorganisms are usually associated with comparatively high levels of L-alanylaminopeptidase activity compared to their Gram-positive counterparts.[1,2] One strategy for detection of L-alanylaminopeptidase activity involves monitoring the increase in fluorescence when a weakly fluorescent substrate **1** is converted into a highly fluorescent heterocyclic amine derivative **2** as depicted by the enzymatic transformation shown in Scheme 1.



Scheme 1. Generation of fluorescence resulting from L-alanylaminopeptidase activity (Het = heteroaromatic).

We have previously reported the synthesis and evaluation of fluorogenic enzyme substrates **3** (X = O, S),[3] and derivatives of these compounds, for the detection of L-alanylaminopeptidase activity in a selection of clinically important microorganisms in Columbia agar media (Figure 1). Good growth of the Gram-negative microorganisms was observed on this medium and, as anticipated, fluorescent colonies were produced by hydrolysis of these substrates. In comparison, the growth of the Gram-positive microorganisms on the medium was either reduced or even inhibited by the substrate, and consequently only weakly fluorescent colonies were produced where microorganism growth had occurred. The fluorogenic substrate **4** was also prepared but, disappointingly, it only gave moderately fluorescent colonies with Gram-negative microorganisms.[4] Thus, we were interested in preparing and assessing whether heterocyclic systems related to structure **4** would produce more intensely fluorescent colonies with Gram-negative microorganisms. The quinoline derivatives **5a-c** and the coumarin derivatives **6-8**, in which the central 1,4-disubstituted phenyl ring in structure **4** is replaced by a 2,6-disubstituted quinoline ring and a 3,7-disubstituted coumarin ring respectively, were therefore chosen for study as potential substrates for the detection of L-alanylaminopeptidase activity in microorganisms. It was anticipated that hydrolysis of these substrates would produce the corresponding fluorescent amine derivatives **9a-c** (from quinolines **5a-c** respectively) and amines **10-12** (from the coumarins **6-8** respectively) (Figure 2). An *N,N*-disubstituted derivative of the amine **9c** has been previously incorporated into a two-photon fluorescent probe for nitric oxide detection in cells and tissues.[5] Related to amines **9**, are derivatives of 2-amino-6-hetarylnaphthalenes (general structure **9**, quinoline nitrogen = CH) which have been prepared and derivatised for a variety of fluorescence applications.[6] 7-Aminocoumarin derivatives are well known fluorescent heterocycles e.g. the L-alanyl derivatives of 7-amino-4-methylcoumarin[7] and 7-amino-4-trifluoromethylcoumarin[8] are substrates that have been utilised for the detection of L-alanylaminopeptidase activity. The aminocoumarin **10** has been described in the literature as an intermediate in the preparation of the corresponding isothiocyanate (**10**, NH_2 = NCS).[9]

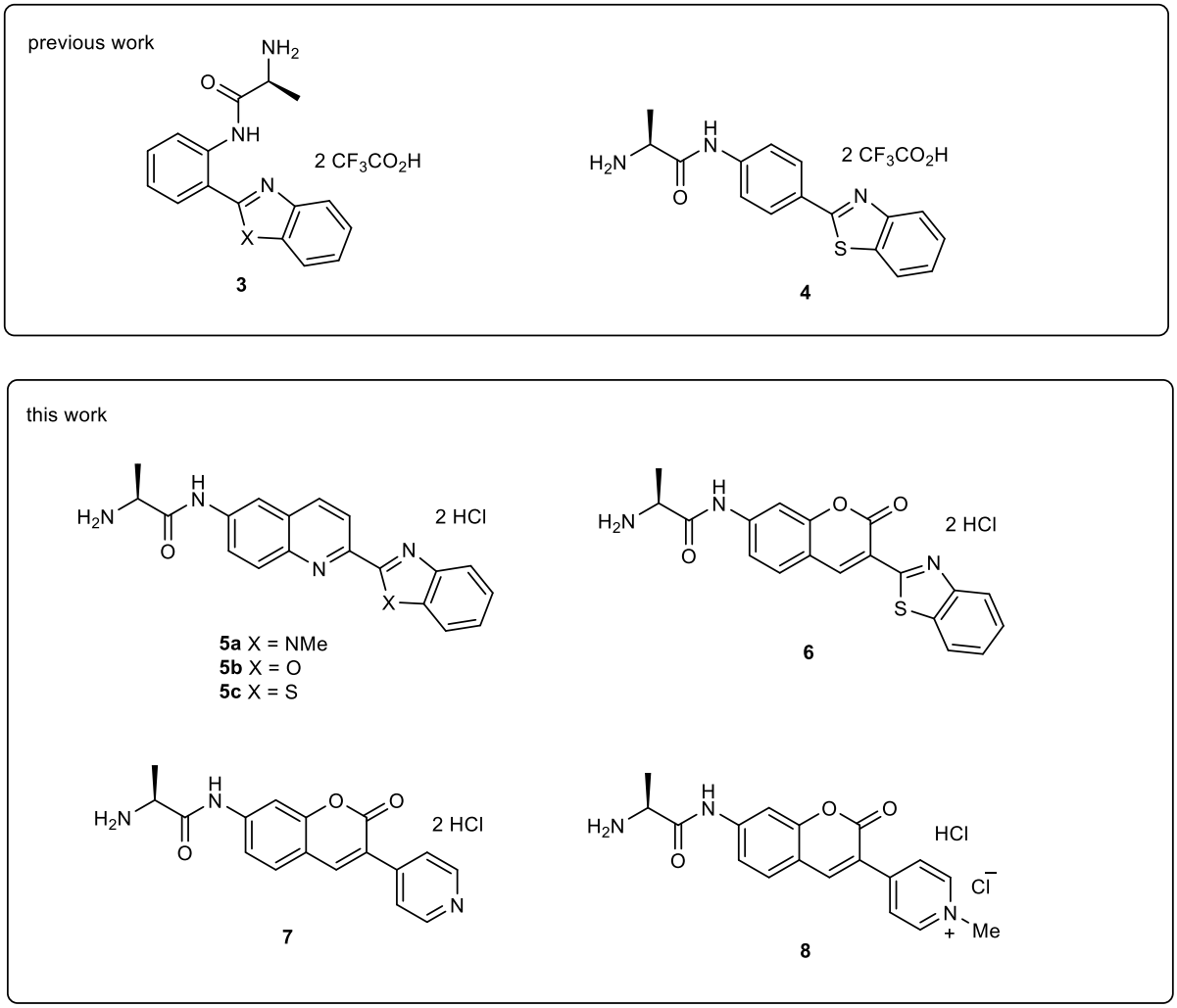


Figure 1. Fluorogenic L-alanylaminopeptidase substrates.

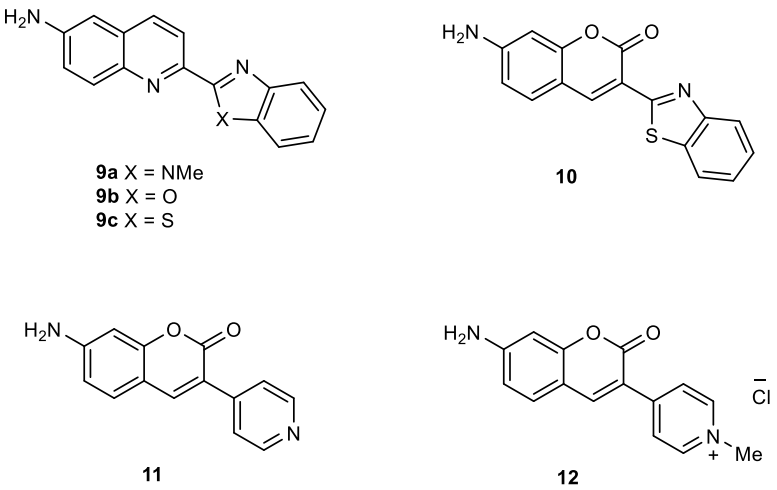
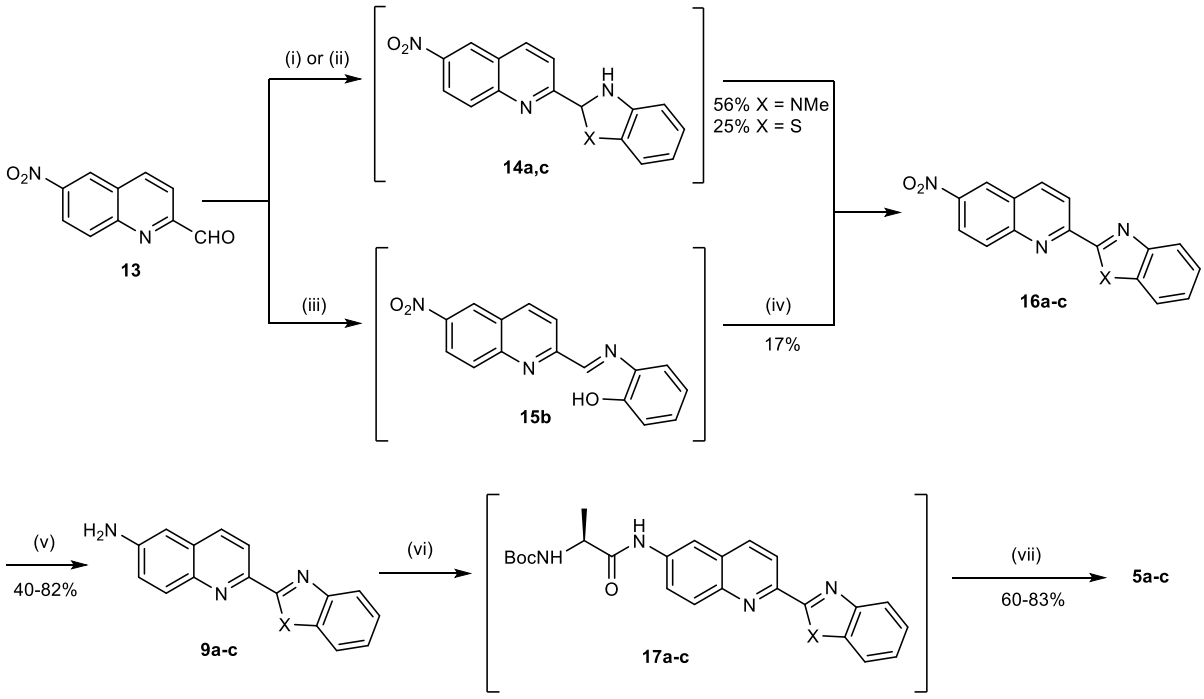


Figure 2. Fluorescent amines **9a-c** and **10-12** resulting from the hydrolysis of substrates **5a-c** and **6-8** respectively.

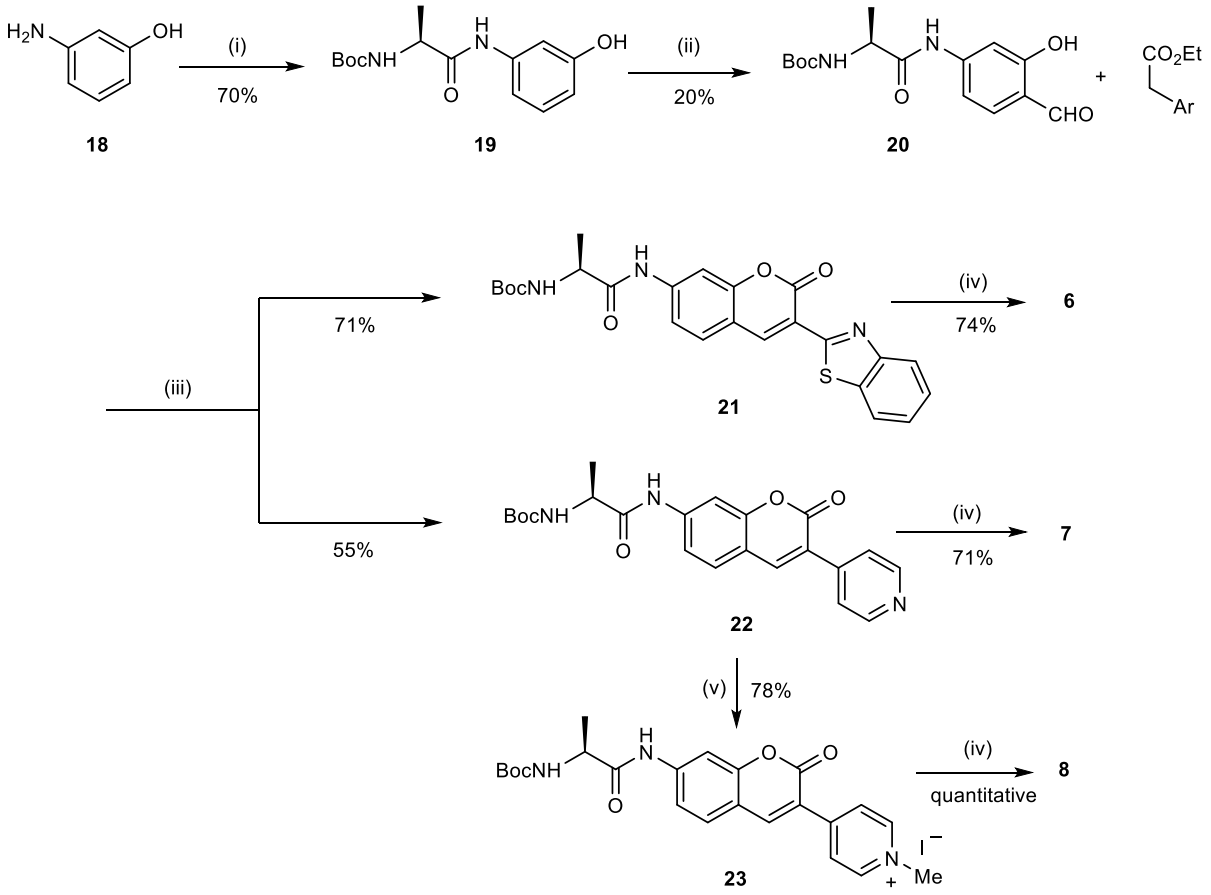
The synthesis of the required series of substrates **5a-c** is depicted in Scheme 2. 6-Nitroquinoline-2-carboxaldehyde **13**[10] was treated with *N*-methyl-1,2-phenylenediamine in the presence of Oxone® giving the nitroquinoline derivative **16a** *via* oxidation of the initially formed dihydro-derivative **14a**. Heterocycle **13** and 2-aminothiophenol in hot dimethylsulfoxide solution produced the benzothiazole derivative **16c** *via* the dihydro intermediate **14c**. The reaction of aldehyde **13** with 2-aminophenol gave a Schiff base **15b** which was subjected to an oxidative cyclisation when treated with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) producing the benzoxazole derivative **16b**. All three nitroquinolines **16a-c** were reduced with tin(II) chloride affording the amines **9a-c** respectively. These amines were then reacted with Boc-L-alanine in a mixed anhydride condensation reaction and the resulting the Boc-protected amides **17a-c** were deprotected using a saturated solution of hydrogen chloride in ethyl acetate furnishing the required substrates **5a-c**.



Scheme 2. Synthesis of the substrates **5a-c**. Reagents and conditions: (i) *N*-methyl-1,2-phenylenediamine, Oxone®, DMF/H_2O (25:1), rt, 16 h; (ii) 2-aminothiophenol, DMSO, 110 °C, 16 h; (iii) 2-aminophenol, EtOH, reflux, 16 h; (iv) DDQ, CH_2Cl_2, rt, 16 h; (v) $SnCl_2.2H_2O$, EtOH, reflux, 16 h; (vi) Boc-L-alanine, *N*-methylmorpholine, isobutyl chloroformate, THF, -5 °C then add **9a-c**; (vii) sat. HCl/EtOAc, rt, 2 h.

The synthetic route adopted for the preparation of substrates **6-8** is outlined in Scheme 3. 3-Aminophenol **18** was subjected to a mixed anhydride coupling reaction with Boc-L-alanine giving the phenol derivative **19**. Compound **19** was then reacted with paraformaldehyde in the presence of $MgCl_2$ under basic conditions[11] producing the salicylaldehyde derivative **20**. The reaction of compound **20** with the appropriate ethyl ester derivatives of hetarylacetic acids under basic conditions afforded the hetarylcoumarins **21** and **22**. Treatment of coumarin **22** with methyl iodide furnished the pyridinium salt **23**. Removal of the Boc protecting-group from compounds **21-23** was

achieved by treatment with a saturated solution of hydrogen chloride in ethyl acetate giving the required substrates **6-8**.



Scheme 3. Synthesis of the substrates **6-8**. Reagents and conditions: (i) Boc-L-alanine, *N*-methylmorpholine, isobutyl chloroformate, THF, -5 ^{o}C then add **18**; (ii) paraformaldehyde, Et_3N, $MgCl_2$, CH_3CN, reflux, 48 h; (iii) piperidine (0.6 equiv.), EtOH, reflux, 18 h; (iv) sat. HCl/EtOAc, rt, 2 h; (v) MeI, CH_3CN, rt, 24 h.

Each of the substrates **5a-c** and **6-8** were evaluated in Columbia agar medium (37 ^{o}C in air for 18 hours) against a panel of 20 clinically important microorganisms, including 10 Gram-negative bacteria, 8 Gram-positive bacteria and 2 yeasts. Columbia agar media was selected because each substrate could be evaluated simultaneously against the complete panel of 20 microorganisms on a single plate. The growth of the microorganisms was compared to control plates in which no substrate was present. The Gram-negative microorganisms all exhibited strong growth on the control plates whereas the Gram-positive microorganisms and the yeasts showed only moderate growth.

The fluorescence produced from the hydrolysis of the L-alanylaminopeptidase substrates **5a-c** by the panel of 20 microorganisms is shown in Table 1 and Figure 3 shows a photograph of a representative agar plate depicting the fluorescence produced from substrate **5c**. There was good growth of all the Gram-negative microorganisms in the presence of substrates **5a-c** and strongly fluorescent colonies were observed with nearly all these bacteria. This activity profile is similar to that observed with the commercially available substrate, L-alanyl-7-amino-4-methylcoumarin (data not shown). Growth of

the Gram-positive microorganisms and yeasts in the presence of substrates **5a** and **5b** was moderate (as in the control plate), with the exception of *Staphylococcus epidermidis*, whose growth was inhibited. In the cases where growth of the Gram-positive microorganisms was apparent, the production of fluorescent colonies was sometimes observed, but the intensity of fluorescence was weak compared to that produced by the Gram-negative microorganisms. Substrate **5c** was inhibitory to the growth of all of the Gram-positive microorganisms as well as the yeasts, hence no fluorescent colonies were generated. Thus, only the Gram-negative microorganisms produced fluorescence with substrate **5c** and this is clearly seen in the photograph of the agar plate shown in Figure 3.

		5a		**5b**		**5c**	
	Microorganism / Reference[a]	**Growth[b]**	**Fluorescence[c]**	**Growth[b]**	**Fluorescence[c]**	**Growth[b]**	**Fluorescence[c]**
	Gram-negative microorganisms						
1	*Escherichia coli* NCTC 10418	++	++ blue	++	++ green	**++**	++ blue
2	*Klebsiella pneumoniae* NCTC 9528	++	++ blue	++	++ green	**++**	++ blue
3	*Providencia rettgeri* NCTC 7475	++	++ blue	++	++ green	**++**	++ blue
4	*Enterobacter cloacae* NCTC 11936	++	++ blue	++	++ green	**++**	++ blue
5	*Serratia marcescens* NCTC 10211	++	++ blue	++	++ green	**++**	++ blue
6	*Salmonella typhimurium* NCTC 74	++	++ blue	++	++ green	**++**	++ blue
7	*Pseudomonas aeruginosa* NCTC 10662	++	++ blue	++	++ green	**++**	+ blue
8	*Yersinia enterocolitica* NCTC 11176	++	++ blue	++	++ green	**++**	++ blue
9	*Burkholderia cepacia* NCTC 10743	++	+/- blue	++	+ green	**++**	++ blue
10	*Acinetobacter baumannii* NCTC 12156	++	++ blue	++	++ green	**++**	++ blue
	Gram-positive microorganisms						
11	*Streptococcus pyogenes* NCTC 8306	+	-	+	Tr green	-	-
12	*Staphylococcus aureus* (MRSA) NCTC 11939	+	-	+	-	**-**	-
13	*Staphylococcus aureus* (MSSA) NCTC 6571	+	-	+	-	**-**	-
14	*Staphylococcus epidermidis* NCTC 11047	-	-	-	-	**-**	-
15	*Listeria monocytogenes* NCTC 11994	+	Tr blue	+	Tr green	**-**	-
16	*Enterococcus faecium* NCTC 7171	+	Tr blue	+	+ green	**-**	-
17	*Enterococcus faecalis* NCTC 775	+	Tr blue	+	+ green	**-**	-
18	*Bacillus subtilis* ATCC 9372	+	-	+	-	**-**	-

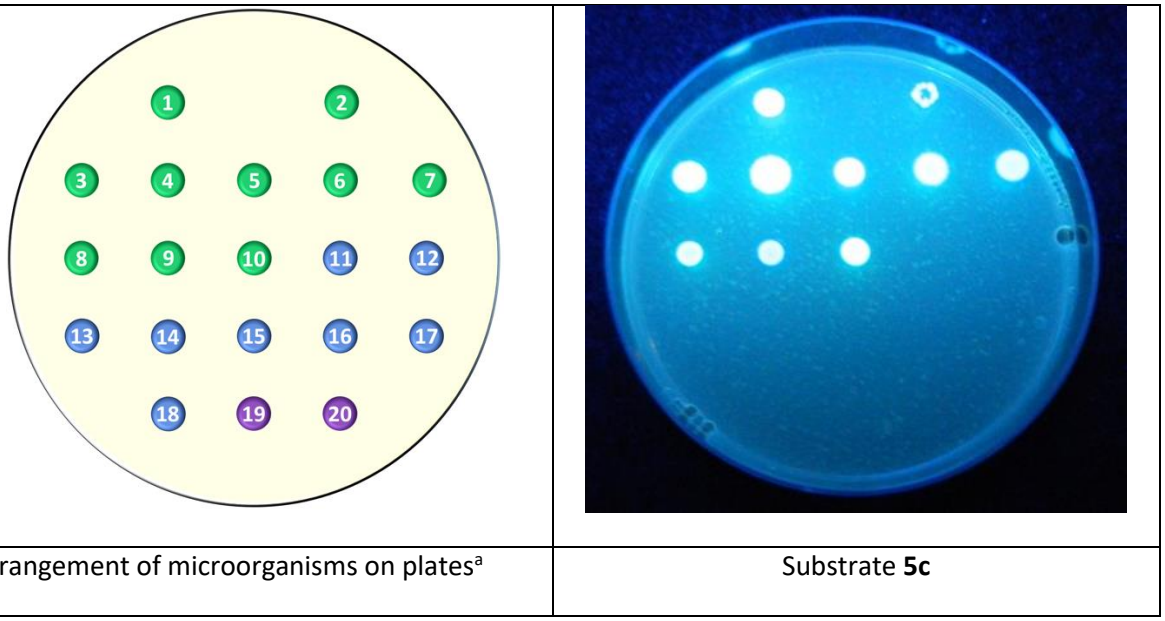
	Yeasts						
19	*Candida albicans* ATCC 90028	+	Tr blue	+	-	-	-
20	*Candida glabrata* NCPF 3943	+	-	+	-	-	-

[a]NCTC: National Collection of Type Cultures; ATCC: American Type Culture Collection; NCPF: National Collection of Pathogenic Fungi.

[b] ++ strong growth, + moderate growth, +/- weak growth.

[c] ++ strong fluorescence, + moderate fluorescence, +/- weak fluorescence, Tr trace of fluorescence.

Table 1. Evaluation of the substrates **5a-c**. Substrate concentration = 100 mg L^{-1}; inoculum = 100 000 colony-forming units (cfu)/spot.



Arrangement of microorganisms on plates[a] | Substrate **5c**

Figure 3. An illustrative Columbia agar plate depicting the fluorescence generated by various microorganisms with substrate **5c**. [a] Microorganisms are numbered in the sequence shown in the Tables. Green spots represent Gram-negative bacteria, blue spots represent Gram-positive bacteria and purple spots represent the yeast species. Plate viewed under UV light (λ = 365 nm).

Good growth of the Gram-negative microorganisms in the presence of substrates **6-8** was observed and moderate growth was noted for the majority of the Gram-positive microorganisms and the yeasts (Table 2). All of the Gram-negative microorganisms produced fluorescent colonies with substrates **6-8** with substrate **6** giving strong fluorescence and substrates **7** and **8** showing moderately intense fluorescence. Only substrate **6** generated moderately intense, fluorescent colonies with some of the Gram-positive microorganisms. There is an interesting contrast between the quinoline-derived substrate **5c** and the coumarin-derived substrates **7** and **8**. Only the Gram-negative microorganisms produced fluorescent colonies with these three substrates; however

substrate **5c** was inhibitory to the growth of Gram-positive microorganisms whereas substrates **7** and **8** were not inhibitory to these microorganisms. Thus, substrates **7** and **8** could potentially be used for differentiation of Gram-negative and Gram-positive microorganisms. Alternatively, for applications that target Gram-negative bacteria specifically, substrate **5c** could potentially be used as a both a fluorogenic indicator and a selective agent for inhibition of Gram-positive bacteria.

A yellow precipitate was noted in the agar plate containing substrate **6**; presumably this substrate has limited solubility in agar media at a concentration of 100 mg L^{-1}. All of the three coumarin substrates **6-8** where therefore evaluated at a concentration of 25 mg L^{-1} and this resulted in broadly similar growth and fluorescence patterns compared to the higher substrate concentration. However, even with this lower substrate concentration, a yellow precipitate was still apparent with substrate **6**. The concentration of the substrate **6** was therefore reduced further to 10 mg L^{-1}. At this substrate concentration, the observed growth and fluorescence was again broadly similar to that at higher concentrations but no substrate precipitation was noted.

		6		**7**		**8**	
	Microorganism / Reference[a]	**Growth[b]**	**Fluorescence[c]**	**Growth[b]**	**Fluorescence[c]**	**Growth[b]**	**Fluorescence[c]**
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9	*Burkholderia cepacia* NCTC 10743	++	++ yellow	++	+ green	++	+/- yellow
10	*Acinetobacter baumannii* NCTC 12156	++	++ yellow	++	+ green	++	+ yellow

	Gram-positive microorganisms						
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18	*Bacillus subtilis* ATCC 9372	-	-	+	-	+	-
	Yeasts						
19	*Candida albicans* ATCC 90028	+	-	+	-	+	-
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[b] ++ strong growth, + moderate growth, +/- weak growth.

[c] ++ strong fluorescence, + moderate fluorescence, +/- weak fluorescence.

Table 2. Evaluation of the substrates **6-8**. Substrate concentration = 100 mg L^{-1}; inoculum = 100 000 colony-forming units (cfu)/spot.

In conclusion, all of the six novel fluorogenic substrates described in this paper produced fluorescent colonies with Gram-negative microorganisms in Columbia agar media. The growth of the Gram-negative microorganisms was not inhibited by these substrates. Moderate growth of the majority of the Gram-positive microorganisms and yeasts occurred in the presence of substrates **5a**, **5b** and **6** and fluorescent colonies were observed, but not with all of these microorganisms. Moderate growth of all of the Gram-positive microorganisms and yeast also occurred with substrates **7** and **8** but no fluorescent colonies were produced, thus allowing differentiation between these microorganisms

and the Gram-negative microorganisms. Substrate **5c** was inhibitory to the Gram-positive microorganisms and the yeasts thus creating a clear distinction between these microorganisms and the Gram-negative microorganisms.

Acknowledgements

We thank bioMérieux SA for generous financial support and the National Mass Spectrometry Facility (NMSF) at Swansea University for high resolution mass spectra.

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